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Force-activatable biosensor enables single platelet force mapping directly by fluorescence imaging

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A B S T R A C T

Integrin-transmitted cellular forces are critical for platelet adhesion, activation, aggregation and contraction during hemostasis and thrombosis. Measuring and mapping single platelet forces are desired in both research and clinical applications. Conventional force-to-strain based cell traction force microscopies have low resolution which is not ideal for cellular force mapping in small platelets. To enable platelet force mapping with submicron resolution, we developed a force-activatable biosensor named integrative tension sensor (ITS) which directly converts molecular tensions to fluorescent signals, therefore enabling cellular force mapping directly by fluorescence imaging. With ITS, we mapped cellular forces in single platelets at 0.4 µm resolution. We found that platelet force distribution has strong polarization which is sensitive to treatment with the anti-platelet drug tirofiban, suggesting that the ITS force map can report anti-platelet drug efficacy. The ITS also calibrated integrin molecular tensions in platelets and revealed two distinct tension levels: 12–54 piconewton (nominal values) tensions generated during platelet adhesion and tensions above 54 piconewton generated during platelet contraction. Overall, the ITS is a powerful biosensor for the study of platelet mechanobiology, and holds great potential in antithrombotic drug development and assessing platelet activity in health and disease.

1. Introduction

Platelets are small anucleate blood cells which aggregate and form the foundation of blood clots during hemostasis and thrombosis (Zucker and Nachmias, 1985). Membrane receptors such as von Willebrand factor receptor (GPIb-V-IX), integrin $\alpha_2\beta_1$ (glycoprotein Ia-IIa), integrin $\alpha_5\beta_1$ (glycoprotein Ic-IIa) and integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) (Bennett et al., 2009; Moser et al., 2008; Rivera et al., 2009) play critical adhesive and regulative roles in platelet physiological functions (Gibbins, 2004). Among the many adhesion receptors in platelets, integrin $\alpha_{IIb}\beta_3$ is the prominent one mediating platelet adhesion under low shear conditions, and is also the main integrin responsible for clot retraction (Moroi and Jung, 1998). When platelets are activated by contact with ligands on the exposed subendothelium in injured blood vessels or by soluble factors including ADP (Adenosine diphosphate) and thrombin, platelet integrin $\alpha_{IIb}\beta_3$ in turn becomes activated allowing it to bind to a variety of ligands including von Willebrand factor, fibrinogen, fibronectin and vitronectin. Activated integrin $\alpha_{IIb}\beta_3$ ultimately mediates platelet adhesion, spreading, aggregation and blood clot retraction (Li et al., 2010). Being indispensable in hemostasis and thrombosis (Bennett, 2005), Integrin $\alpha_{IIb}\beta_3$ is known to transmit cellular forces which are essential for the retraction and stabilization of the blood clot and thrombus (Liang et al., 2010; Rooney et al., 1998). Therefore, measuring, visualizing and mapping integrin $\alpha_{IIb}\beta_3$-transmitted platelet forces would provide valuable biomechanical information for the understanding of platelet function mechanisms and for the assessment of platelet activities. However, these forces have never been measured at the molecular level or mapped at submicron resolution.

Previously, multiple techniques including cell traction force microscopy (Henriques et al., 2012), atomic force microscopy (Lam et al., 2011; Lee and Marchant, 2001), elastomers (Myers et al., 2017; Qiu et al., 2014) and micro-post assays (Feghhi et al., 2016; Liang et al., 2010) have been applied to the bulk force measurement in individual platelets. These pioneering methods have enabled the measurement of bulk platelet forces and advanced the study of platelet mechanobiology

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to a single cell level. These methods commonly adopted the force-to-strain approach for cellular force visualization and measurement: using elastic substrates with a pre-calibrated modulus to convert platelet forces to a visible and measurable substrate strain which is in turn used to compute cellular forces. While powerful at measuring the whole cellular forces, the force-to-strain approach typically yields a low resolution of about 2–5 μm in cellular force imaging (Polacheck and Chen, 2016). This resolution is not ideal for force mapping in single platelets because platelets are small, averaging about 2–4 μm in diameter (Paulus, 1975). As a result, platelet forces have not been mapped with an ideal resolution and very little is known about platelet force distribution at the subcellular level. Besides force mapping, molecular tensions transmitted by single integrins have never been calibrated in platelets. However, integrin tensions are the fundamental mechanical signals driving integrin signaling pathways which are essential for platelet functions (Chicurel et al., 1998). Thus to further the understanding of how platelets generate force and how the force in turn regulates platelet functions, it is imperative to study platelet forces at the molecular level.

To calibrate and map molecular tensions transmitted by integrin $\alpha_{IIb}\beta_3$ in platelets, we developed a force-activatable biosensor named integrative tension sensor (ITS). Instead of converting force to strain, the ITS directly converts molecular tensions to fluorescent signals (force-to-fluorescence conversion). As a molecular linker, the ITS is initially non-fluorescent and can be permanently activated to fluoresce by a tension. The tension threshold required for ITS activation is tunable in the range of 10–60 piconewton (pN), therefore enabling the selective visualization of molecular tensions at different force levels. On a surface where integrin ligands are tethered by the ITS, integrin tensions in platelets activate the ITS and can be directly mapped by fluorescence imaging without the need of post modeling and computation which is usually required by force-to-strain approaches. The force mapping resolution is determined by fluorescence microscopy and easily reaches the submicron level. Using the ITS, for the first time, we mapped integrin-transmitted platelet forces with submicron resolution (0.4 μm) and revealed the polarized cellular force distribution in single platelets. The integrin tension map was then tested to assess the change in platelet activation state in response to an anti-platelet drug tirofiban. Using the ITS, we also calibrated integrin tensions in platelets and revealed two distinct tension levels. We found that platelets produce low-level integrin tensions (12–54 pN. nominal values) during adhesion, while the tensions rise to a high level (> 54 pN. nominal values) during contraction. This work paves the road for the study of mechanobiology of platelets at the molecular level by visualizing, calibrating and mapping integrin tensions, and demonstrates the potential of the ITS as a high-resolution force mapping tool for platelet function evaluation and anti-platelet drug assessment.

2. Materials and methods

2.1. ITS synthesis

ITS is a double stranded DNA (dsDNA) with 18 base pairs. The single-stranded DNAs for ITS synthesis were all customized and purchased from IDTDNA. Their sequences and modifications are shown below. ThioMC6-D/ denotes the thiol conjugation to the 5′ end of DNA. /BiosG/ and /Bio/ denote the DNA modifications with biotin conjugations at 5′ end and 3′ end, respectively. BHQ2 is the black hole quencher 2. The DNA sequences were selected and analyzed by DNA analysis tool available in IDTDNA company website to minimize the probability of self-dimer and hairpin formation in the ssDNA.

5′- /ThioMC6-D/GGG CGG CGA CCT CAG CAT/BHQ2/ -3′
5′- /BiosG/T/iCy3/ATG CTG AGG TCG CCG CCC/ -3′
5′- /Cy3/ATG CTG AGG TCG CCG CCC/Bio/ -3′
5′- /Alexa647/ATG CTG AGG TCG CCG CCC/Bio/ -3′

Cyclic peptide RGD with an amine group (PCI-3696-PI, Peptides International) was conjugated with the DNA strand with thiol modification. The Cyclic RGD has a PEG linker which enhances the accessibility of the RGD to integrins on cell membrane. Conjugation was conducted according to a previously published protocol (Wang and Ha, 2013). Briefly, the thiol modification on 5’ end of DNA was deprotected by TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) and reacted with the maleimide group of a heterolinker SMCC-sulfo (22622, thermos scientific). The other end of SMCC-sulfo is a NHS ester (N-hydroxysuccinimide esters) which reacts with the amine of RGD. The RGD conjugated DNA was purified by electrophoresis. The 12 pN and 54 pN ITSs used in this paper were assembled by hybridizing the two ssDNAs with a concentration ratio of 1.1:1 (The strand with quencher: the DNA strand with fluorophore).

ITS with 54 pN threshold (with Cy3-BHQ2 pair)
5′- /RGD/GGG CGG CGA CCT CAG CAT/BHQ2/ -3′
5′- /BiosG/T/iCy3/ATG CTG AGG TCG CCG CCC/ -3′
ITS with 12 pN threshold (with Cy3-BHQ2 pair)
5′- /RGD/GGG CGG CGA CCT CAG CAT/BHQ2/ –3′
5′- /Cy3/ATG CTG AGG TCG CCG CCC/Bio/ -3′
ITS with 12 pN threshold (with Alexa647-BHQ2 pair)
5′- /RGD/GGG CGG CGA CCT CAG CAT/BHQ2/ -3′
5′- / Alexa647/ATG CTG AGG TCG CCG CCC/Bio/ -3′

2.2. Preparation of the ITS coated surface

The ITS surface yields a strong fluorescent signal due to the signal integration process, leading to a high tolerance for surface defects or fluorescence background. Moreover, the ITS immobilization starts with physical adsorption of bovine serum albumin-biotin on a surface. These two features enable the ITS to be coated directly on regular cell culture surfaces. In ITS surface preparation, glass-bottom dish (D35-10-1.5-N, In Vitro Scientific) or glass-bottom 96-well plate (P96G-0–5-F, MaTek Corp) was first immersed in 100 μg/ml BSA-biotin (biotinylated bovine serum albumin, A8549, Sigma-Aldrich) mixed with 5 μg/ml fibronectin for 30 min at room temperature, followed by three PBS washes. The fibronectin facilitates platelet adhesion and minimizes the influence of the ITS rupture on platelet activities. The dish was then incubated with 100 μg/ml neutravidin (31000, ThermoFisher Scientific) for 30 min at room temperature. Neutravidin is now immobilized on the surface through avidin-biotin interactions. After being washed with PBS three times, the dish was coated with 0.1 μM ITS-biotin and incubated for 30 min at 4 °C. At this stage, the ITS is immobilized on the neutravidin surface (each neutravidin molecule has four biotin binding sites) with the binding scheme: glass:BSA-biotin:neutravidin:biotin-ITS. After being rinsed with PBS, the ITS surface is ready for platelet seeding and force mapping.

2.3. Preparation of platelet-rich plasma (PRP)

The ITS methodology was developed using canine platelets because we had ready access to canine blood. Blood was drawn from healthy research dogs with permission from Iowa State University's Institutional Animal Care and Use Committee. During a typical ITS assay, 1.8 ml blood was drawn from a dog's cephalic vein into a 3-ml syringe containing 0.2 ml Acid Citrate Dextrose (ACD) buffer (ACD buffer: 85.3 mM sodium citrate, 41.6 mM citric acid, 136 mM glucose), and then transferred into a 15-ml falcon tube containing 2 ml buffered saline glucose citrate (BSGC: 129 mM NaCl, 14 mM Na_3citrate, 11 mM glucose, 10 mM NaH_2PO_4, pH 7.3). The mixture was centrifuged at 180*g* for 8 min at room temperature. The supernatant was harvested as platelet-rich plasma (PRP) and kept at room temperature until future use.

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